EXHIBIT 1

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US006686191B1

(12) **United States Patent**
Alonso

(10) Patent No.: **US 6,686,191 B1**
(45) Date of Patent: **Feb. 3, 2004**

(54) PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1772 days.

(21) Appl. No.: 08/532,211

(22) Filed: **Sep. 22, 1995**

(51) Int. Cl.^{7} C12N 7/04; A61K 39/395; A61K 39/40; A61K 39/42

(52) U.S. Cl. 435/236; 424/176.1; 424/177.1; 424/130.1

(58) Field of Search 530/390.1, 390.5, 530/386, 387.1; 424/176.1, 177.1, 130.1; 435/236

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4,396,608 A * 8/1983 Tenold
4,540,573 A * 9/1985 Neurath et al.
4,762,714 A * 8/1988 Mitra et al.

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Joy Yang, Y.H. et al. "Antibody Fc functional activity of intravenous immunoglobulin preparations treated with solvent–detergent for virus inactivation" Vox Sang, vol. 67, pp. 337–344, May 17, 1994).*

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(57)   **ABSTRACT**

Method of reducing the anticomplement activity (ACA) resulting from viral inactivation treatment of a solution of antibodies, the method comprising contacting the solution with a trialkylphosphate, such as tri-n-butyl phosphate, and a detergent, such as sodium cholate, under conditions sufficient to reduce substantially the virus activity, and then incubating the solution under controlled conditions of time, pH, temperature, and ionic strength such that the anticomplement activity is reduced to an acceptable level. In a preferred embodiment, the ACA is reduced to less than 60 CH_{50} units/mL, the incubation is for at least about ten days at a pH from 3.5 to 5.0, the temperature is maintained within a range of 2 to 50° C., and the ionic strength of the solution is less than about 0.001 M.
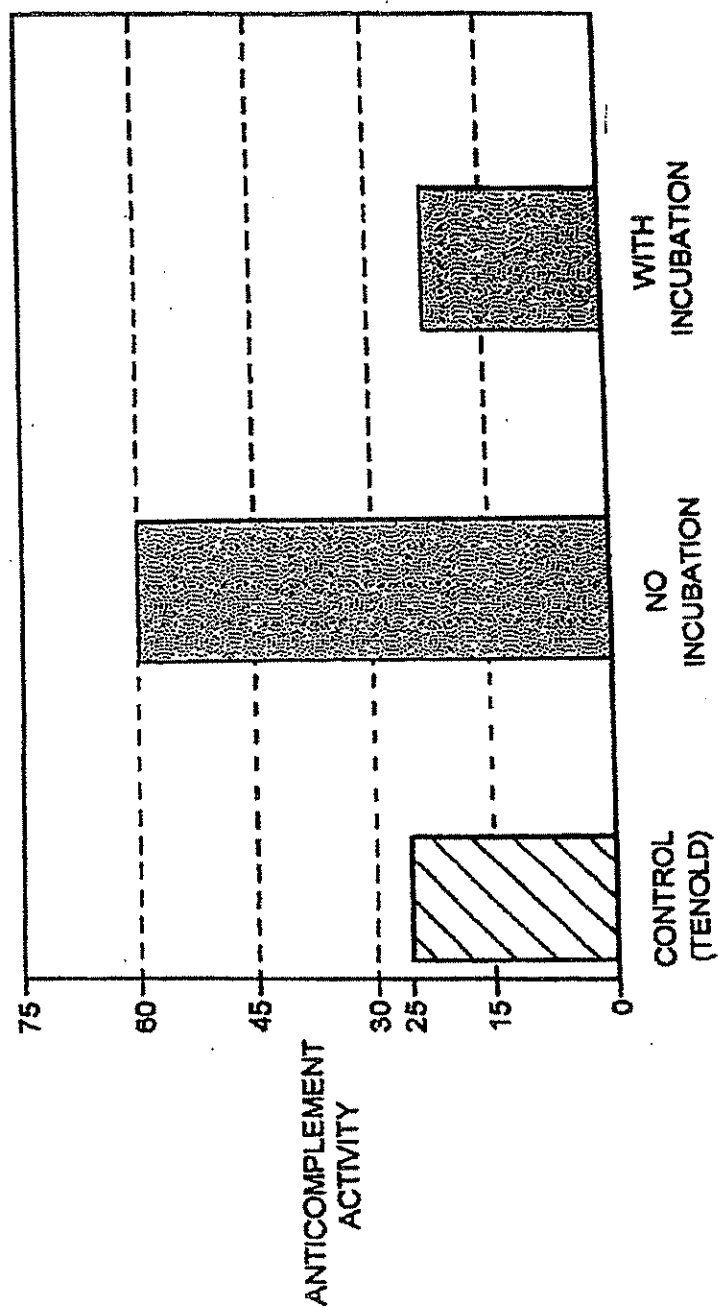
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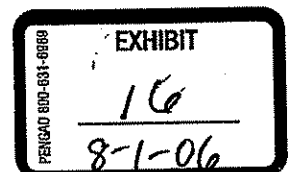
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PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

BACKGROUND OF THE INVENTION

1. Field

This invention generally deals with an intravenously injectable immunoglobulin product, and more specifically deals with an intravenously injectable immune serum globulin (IGIV) which has been subjected to a virus inactivation step and which has a low level of anticomplement activity.

2. Background

Early pharmaceutical preparations of immune serum globulins could not be administered intravenously due to an unacceptably high incidence of adverse reactions. These adverse reactions were associated with a decrease in serum complement levels, apparently caused by complement binding to the administered gamma globulin. (1) The ability of gamma globulin to bind complement, or its anticomplement activity (ACA), is greatly increased as a result of denaturation brought about during the fractionation procedure. Several approaches have been taken to address the problem of rendering ISG safe for intravenous administration. (See (2) and references therein). Tenold reported a method of preparing an immune serum globulin (ISG) with low ACA which could be administered by intravenous injection. (2, incorporated herein by reference). The Tenold '608 process requires formulating the ISG at low ionic strength (preferably less than about 0.001) and at low pH (3.5–5.0).

Other methods of preparing intravenously injectable immune serum globulin (IGIV) have been reported, including stabilizing with carbohydrates such as maltose (3). A process including incubation of ISG at pH 4.0 at 37° C. (4) results in a product with low ACA which may be administered by intravenous injection; however, upon storage the product regains its high ACA. IGIV has also been prepared by covalent modification of the ISG, for example by proteolysis (5) or by reduction of disulfide linkages followed by reaction with a blocking agent (1,6).

Antibody preparations, since they are isolated blood products, have an inherent hazard of transmitting virally-mediated diseases. Inactivation of viruses is an important step in producing safe and effective blood products. U.S. Pat. No. 4,540,573 to Neurath et al., which is incorporated herein by reference, describes a viral inactivation process using a trialkyl phosphate and detergent process (hereinafter, the solvent/detergent process, or SD process). (7) That solvent/detergent method has gained acceptance as being efficacious in the inactivation of lipid-enveloped viruses with limited adverse effects on biological activity or blood product profile. (8, 15; See also 12 for a discussion of various viral inactivation processes).

Current antibody preparations on the market generally have been regarded as safe with respect to viral contamination. (9) This is thought to be due to features of the fractionation processes used to isolate these blood products. However, it would be desirable to further ensure the safety of the antibody preparations by including a distinct viral inactivation step in the production process. Successful reduction of viral activity in an IGIV solution was reported using several different methods of viral inactivation for a variety of viruses. (16, 17) A process for preparation of immunoglobulins substantially free of retrovirus has been reported involving incubation of ISG under controlled conditions of time, temperature, and pH. The process entails

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isolating ISG via a cold ethanol plasma fractionation process and then storage of the ISG at one of two storage conditions: (a) at pH≤4.25 at a temperature of 27° C. for at least three days, or (b) at pH≤6.8 at a temperature of 45° C. for at least six hours. (10).

We have found that using the SD process to treat ISG preparations, especially those subsequently formulated according to the Tenold '608 patent, results in a product with an acceptable viral inactivation but with unacceptably high levels of ACA. Elevated ACA levels were always detected at the sterile bulk stage (i.e., after compounding as 5% or 10% IGIV and filtration with 0.2 μm sterile filters) of all tri-n-butyl phosphate (TNBP)/detergent treated IGIV preparations regardless of process scale. Preparations of ISG with high ACA levels are not suitable for intravenous injection and instead must be administered via other routes, e.g. intramuscular (IM) injection. However, IGIV preparations are more desirable since they are immediately available in the bloodstream and are not subject to loss associated with IM injection. It is thus desirable to have an IGIV product which is both low in ACA and has been subjected to a viral inactivation step.

SUMMARY OF THE INVENTION

The invention is a method for producing an intravenously injectable immune serum globulin (IGIV) preparation with low anticomplement activity which has been chemically treated to render it substantially free of lipid-enveloped viruses. The method comprises a solvent/detergent viral inactivation step followed by an incubation step. We have discovered that the incubation step is necessary to achieve an acceptable level of ACA low enough to allow the ISG to be administered by intravenous injection. The incubation step should be conducted under controlled time, pH, temperature, and ionic strength. Preferably, the pH should be maintained between about 3.5 and about 5.0, the temperature should be within a range of about 2 to about 50° C., and the ionic strength should be less than about 0.001M. In a preferred embodiment the ACA of the ISG preparation decreases gradually over a period of at least about ten days when the ISG is maintained at a pH of about 4.25 at low ionic strength (less than about 0.001M) and the viral inactivation step (in a model system) results in a substantial reduction (i.e. at least 4 logs) in the titer of lipid enveloped viruses.

BRIEF DESCRIPTION OF THE FIGURE

FIG. 1 shows a comparison of the typical average observed ACA levels of 5% IGIV solutions treated according to the SD process and with or without the follow-up incubation of the present invention.

SPECIFIC EMBODIMENTS

Materials and Methods

The starting material for the process of this invention is unmodified human immune serum globulin. In the specification and claims the term "immune serum globulin" is used to define the substance also referred to in the literature variously as gamma globulin, IgG and immunoglobulin G. It consists predominantly and preferably of at least about 85 percent of the 7S species of gamma globulin, which has a molecular weight of about 160,000. Any remainder is preferably 9S species, with a molecular weight of about 300,000. Both standard immune and hyperimmune serum globulins, e.g., tetanus, rabies and hepatitis immune serum globulins,

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can be employed, the solvent/detergent treated product being immune and hyperimmune ISG, respectively. Thus, a suitable starting material for the process of this invention is Cohn's Fraction II or Fraction III filtrate. (See Refs. 13, 14.)

Fraction II, by ultracentrifugation studies, is predominantly (about 85 percent) the 7S (sedimentation constant of 7) species of gamma globulin with an average molecular weight of 160,000. The remaining protein is essentially 9S material with a M.W. of about 300,000. Wet Fraction II paste (approximately 30 percent solids) is commonly lyophilized to obtain dry ISG powder which is then dissolved and prepared for intramuscular injection as a 16.5 percent sterile solution. Either the wet Fraction II paste or the dry ISG powder is a suitable starting material for the process of this invention.

Gamma globulin obtained by any process which has essentially the same composition of protein components as found in the Cohn Fraction II or Fraction III filtrate can be used as starting material in the present process. Both standard immune serum globulin and hyperimmune serum globulin can be employed as starting materials. As is well known, the latter is produced from plasma or serum obtained from selected donors who have much higher titers for a specific antibody than is normally found in the average population. These donors have either been recently immunized with a particular vaccine or else they have recently recovered from an infection or disease. These high titer sera or plasmas are pooled and subjected to the usual Cohn fractionation procedures up to the point of isolating Fraction II.

Furthermore, because the amount of antibody required to achieve a desired immunological response is substantially less when administered intravenously, it will be apparent the intravenous dose will be substantially less than the intramuscular dose which will produce the same serum antibody titer. Thus, the dose of intramuscular ISG and hyperimmune serum globulin must be higher than that required to achieve the same serum antibody titer when globulin of the same antibody activity is administered intravenously.

The starting wet paste or lyophilized powder is dissolved in a volume of water or other physiologically-acceptable carrier to provide a protein solution of a concentration of about 0.5–20% preferably about 5 to 10 percent. If Fraction III filtrate is employed, the aqueous solution must be concentrated by conventional techniques to the desired protein concentration. Any protein concentration may be used in this method; however, the above range is preferred from a practical standpoint.

After the protein has been dissolved or concentrated, the solution is adjusted to a pH of about 3.5 to 5.0 preferably about 3.8 to 4.2, by addition of a physiologically-acceptable acid such as hydrochloric acid. In general, the pH is adjusted to a point whereat the monomeric material in the protein solution is maintained at a maximum. However, the pH must not be so low as to result in gelation. The temperature should not be harmful to the ISG material. Good results are obtained within the temperature range of about 0–20° C. It is not necessary to hold the so-adjusted material for any period of time prior to the next step; however, the material may be held, if desired, without detrimental effects.

The protein solution at the appropriate pH (preferably 3.8–4.2) may be diafiltered with at least 4 volume exchanges of water to reduce the alcohol concentration from approximately 17% (Filtrate III) to about 2% alcohol. The efficacy of solvent/detergent as a viral inactivation method is much better at or above ambient temperatures; however, high

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concentrations of alcohol at these temperatures will denature the IgG molecules. Thus, this inactivation must be performed in low alcohol concentration.

Next, the protein concentration of the so-treated material is adjusted to the level desired for incubation with TNBP/detergent, generally less than 10% protein for maximum viral inactivation. This adjustment is accomplished by conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Prior to addition of TNBP/detergent, the pH may be adjusted within a wide range, depending on the detergent to be used. With Tween 80, the pH may be as low as 3.5, where the IgG starts becoming unstable. With cholate, the pH is adjusted to within the range of 5.0–6.4, preferably about 5.6, prior to addition of TNBP/detergent. Satisfactory cholate solubility during incubation was achieved by adjusting the immunoglobulin solutions to a pH of 5.5 or higher prior to addition of TNBP and sodium cholate. Adjusting the IgG solution to pH values lower than 5.5 is not suitable because the solubility of sodium cholate is highly dependent on pH (cholic acid pK=6.4), with poor solubility at pH 5.5 or lower. Furthermore, maximum viral inactivation during incubation with TNBP/cholate was observed at pH values less than 6.0 in experiments which employed model viruses spiked into IgG solutions. The inactivation of HIV-1 and BVDV (bovine viral diarrhea virus, which is employed as a model for hepatitis C) was accelerated at pH 5.8, with inactivation to the detection limit occurring in 1–2 hours, whereas inactivation to the detection limit required a minimum of 6 hours when pH 7 conditions were used.

Next, the TNBP/detergent is added to the protein solution (preferably less than 8% [w/w], pH 5.8) mixed thoroughly, and then incubated above ambient temperatures, for example 30° C., with continuous agitation or mixing. Target TNBP/cholate levels for optimal viral inactivation during the incubation step should be >3 mg/mL TNBP and >2 mg/mL cholate as defined by Edwards et al. (8) Moreover, for effective viral inactivation, it is important that the solution is essentially free of particulates in order to facilitate thorough mixing of solvent/detergent and IgG solution. After incubation with TNBP/cholate under these conditions, greater than 5.2 $\log_{10}$ reduction of HIV-1 and greater than 4.0 $\log_{10}$ reduction of BVDV were detected.

After completing the incubation which provides the viral inactivation, the solvent and detergent molecules must be removed in order to achieve a final product with low levels of residual TNBP and cholate which would be suitable for intravenous administration. Generally, procedures to remove detergent are also effective in removing TNBP, and vice versa. Very low levels of TNBP and cholate in the final container can be achieved by a combination of filtration, diafiltration and hydrophobic chromatography. After completing the incubation, the majority of cholate (and TNBP) can be removed from the protein solution by filtration, providing the solution had been previously adjusted to a lower pH value such as 4.0, because sodium cholate is sparingly soluble in aqueous solutions at such pH values. Moreover, all processing steps which follow the solvent/detergent incubation are performed at lower pH values (i.e., 4.0) because IgG molecules are more stable at pH values between 3.5–5.0, in low ionic strength solutions. (2) Thus, after incubation with TNBP/cholate, the protein solution is adjusted to approximately pH 4.0 and incubated at 0–8° C. in order to promote cholate precipitation. Next, filtration is employed to remove the precipitated cholate from the IgG solution.

The so-treated solution is diafiltered with at least four volume exchanges of water to reduce the ionic strength and

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to remove additional TNBP and cholate. After or during the above treatment, the pH is measured and maintained within the range of about 3.5–5.0. The protein concentration of the so-treated material is adjusted to 10–30%, usually 13% (w/v) by employing conventional techniques not detrimental to ISG, e.g., ultrafiltration, reverse osmosis, sublimation, evaporation, etc. Again the pH of the preparation is maintained within the range of about 3.5–5.0, preferably about 3.8–4.2.

In the present invention, hydrophobic chromatography is employed to remove the TNBP and cholate not eliminated by the filtration and diafiltration steps, and thus provide a final product with low levels of residual TNBP and cholate which is suitable for intravenous administration. Hydrophobic chromatography is a method for TNBP removal from protein solutions that has fewer drawbacks and limitations than other available methods such as oil extraction, ion exchange or affinity chromatography. In part, this is because the protein of interest (IgG) remains in solution throughout the TNBP removal process. Polystyrene-based resins (typically PLRP-S from Polymer Laboratories, Amherst, Mass.) were used to remove the solvent/detergent from solution, as we have found the polystyrene-based resins to be superior to other resins, such as silica-based C-18 resins.

Next, the ISG preparation is adjusted to 5% or 10% protein, and treated to render it ionic, i.e., to render it compatible with physiological conditions, or render it physiologically acceptable upon injection. In a preferred embodiment, the tonicity is adjusted to about 230 to about 490 mosmol/kg solvent. More preferably, the tonicity range is from about 250 to about 350 mosmol/kg solvent, and most preferably the tonicity range is from about 260 to about 325 mosmol/kg solvent. The 5% formulation (5% IGIV) is made tonic by the addition of 10% maltose. The 10% formulation contains 0.2 M glycine in order to achieve an isotonic preparation without large quantities of sugar. The product with either formulation (Gamimune®N 5% or Gamimune®N 10%) experiences shifts in molecular distribution (antibody aggregation) when the ionic strength of the low pH solution is increased. Therefore, sodium chloride, which is often used to achieve tonicity, should not be used.

The so-treated solution is incubated at pH 4.25 under low ionic strength conditions (NLT 21 days at 20–27° C. preferred) in order to provide a lowering of ACA levels. The ionic strength is determined according to Perrin (18), and in a preferred embodiment the ionic strength should be less than about 0.001M. Elevated ACA levels were always detected at this stage of all TNBP/cholate treated IGIV preparations (regardless of process scale); however, ACA levels are gradually lowered by incubation at pH 4.25 under low ionic strength conditions (Tables 3, 5–7). While there is no strict rule for determining when the ACA level is low enough to be an acceptable level suitable for intravenous administration, IGIV preparations should have ACA levels as low as possible.

The Figure depicts the typical average reduction of ACA observed in 5% IGIV solutions following SD treatment. For a 5% ISG formulation the acceptable level suitable for intravenous administration preferably would be less than about 45 CH_{50} units/mL, and more preferably less than about 30 CH_{50} units/mL. For a 10% ISG formulation, the acceptable level suitable for intravenous administration preferably would be less than about 60 CH_{50} units/mL, and more preferably less than about 45 CH_{50} units/mL. As used herein, one unit of ACA activity (one CH_{50} unit) is defined as the amount of protein capable of activating 50% of the complement in an optimally titered complement and red

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blood cell/hemolysin system. The assay measures the amount of complement that is bound by the mixture of standardized amounts of complement and protein. See refs. 19–20 for a discussion of the assay. Briefly, red blood cells that have been sensitized by preincubation with red blood cell antibodies are added to the complement/protein mixture. In the presence of free complement (not already bound by the protein) these sensitized cells will lyse, releasing hemoglobin which can be quantitated as a measure of the degree of lysis. In parallel, sensitized red blood cells are also added to a buffer control-complement mixture, whose degree of lysis is defined as 100%. The difference between the actual amount of complement needed to give 100% lysis and the amount of complement remaining unbound in the presence of protein equals the amount of complement actually bound by the protein, or anticomplement activity.

Results

Anticomplement Activity of ISG Resulting From Viral Inactivation Process

To establish the effect of the SD viral inactivation process on solutions containing ISG which are formulated according to the Tenold '608 patent, the experiments depicted in Table 1 were performed. The starting material (SM) was Cohn process filtrate III which had been ultrafiltered to about 5% protein and then diafiltered with four volumes of water.

In the control experiment, incubation (–)/SD (–), the SM was not subjected to any incubation or solvent/detergent treatment. In the incubation (+)/SD (–) experiment, the pH of the SM was adjusted to 7.0, the solution was incubated at 30° C. for ten hours, and then the pH was reduced to 4.0. In the incubation (+)/SD, TNBP & Tween 80 (+) experiment, the pH of the SM was adjusted to 7.0, 3 mg/mL TNBP and 2 mg/mL Tween 80 were added to the solution, the solution was incubated at 30° C. for ten hours, and then the pH was reduced to 4.0. In the incubation (+)/SD, TNBP & cholate (+) experiment, the pH of the SM was adjusted to 7.0, 3 mg/mL TNBP and 2 mg/mL cholate were added to the solution, the solution was incubated at 30° C. for ten hours, and then the pH was reduced to 4.0. The solutions in each experiment were then diafiltered with four volumes CWFI (cold water for injection) and concentrated by ultrafiltration. After addition of dry maltose to 10% w/v, the 5% IGIV solution (pH 4.25) was filtered through a 0.2 μm filter.

TABLE 1

Anticomplement activity in 5% IGIV produced by variations of the Solvent/Detergent IGIV Process	ACA (CH_{50}/mL)
Control (no solvent/detergent treatment, no 30° C. incubation)	25
Incubate at 30° C. for 10 hr (no solvent/detergent)	22
Incubate at 30° C. for 10 hr NLT 3 mg/mL TNBP NLT 2 mg/mL Tween 80	68
Incubate at 30° C. for 10 hr NLT 3 mg/mL TNBP NLT 2 mg/mL cholate	>100

*These samples were assayed for ACA after final compounding according to the Tenold '608 patent, but they were not incubated at pH 4.25 and 22° C. prior to analysis.

The results listed in Table 1 show that levels of ACA increased in IgG samples after incubation with TNBP/cholate or TNBP/Tween 80. ACA levels were not elevated in IgG samples that were incubated for 10 hr at 30° C. in the

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absence of solvent/detergent. These results suggest that ACA levels of IGIV samples were not elevated by either processing manipulations or incubation for 10 hr at 30° C. in the absence of solvent/detergent.

TABLE 2

Anticomplement activity in 5% IGIV
spiked with TNBP/Na cholate

	ACA (CH$_{50}$/mL)
5% IGIV, no TNBP/cholate	12
5% IGIV with 100 μg/mL TNBP, 100 μg/mL Na cholate	13

Furthermore, spiking experiments (with TNBP and Na cholate, Table 2) have demonstrated that the elevated anticomplement activity levels were not artifacts caused by carrying out the anticomplement assay in the presence of trace levels of TNBP/Na cholate. Thus, using the prior art SD process for viral inactivation of a solution containing ISG, subsequently formulated according to the Tenold '608 patent, yields a product which has high ACA and is unsuitable for intravenous administration. In a similar experiment, SD treated samples which were not incubated (Table 3, Initial Testing) had ACA levels greater than 100 units.

TABLE 3

Reduction in Anticomplement activity of
samples previously treated with TNBP/cholate

	ACA (CH$_{50}$/mL)	
Sample	Initial Testing (no incubation)	After incubation 6 wk. @ 5° C. 3 wk. @ 22° C.
RB21872-16	>100	33
RB21872-17	>100	34
RB21872-18	>100	36
RB21872-20	>100	27

However, when duplicate SD treated samples were incubated for extended periods of time (6 weeks at 5° C. and 3 weeks at 22° C.), the level of ACA was markedly reduced (Table 3, after incubation). This led to further investigation of this surprising observation.

Aggregate Content of ISG Exposed to TNBP/cholate

The samples of the previous experiment (Table 3, Initial Testing) were analyzed by size exclusion (gel permeation) HPLC immediately after compounding to determine the extent of aggregation of the IGIV at the initial time point. HPLC analysis shows nearly complete monomer content in the samples. (Table 4).

TABLE 4

HPLC analysis of non-incubated 5% IGIV samples (Table 3 Initial)

Sample	Aggregate (%)	Dimer (%)	Monomer (%)	Fragment (%)
RB21872-16, initial	0.140	0.00	99.86	0.00
RB21872-17, initial	0.146	0.00	99.85	0.00
RB21872-18, initial	0.124	0.00	99.88	0.00
RB21872-20, initial	0.172	0.00	99.83	0.00

Previously, high IgG aggregate levels were shown to correlate with high anticomplement activity. However, results from analysis of the samples show the level of ACA

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in the samples to be greater than 100 units. (Table 3, 'Initial Testing') The HPLC analysis shows that the high ACA following the TNBP/cholate treatment was not due to the presence of aggregated IgG molecules.

Varied Conditions of Time and Temperature

The SM was the same as in the previous experiment, and experimental conditions were similar with the following changes. The solutions were treated with TNBP/cholate at pH 7.0 and then were compounded to 5% IGIV, 10% maltose, pH 4.25, as above. The ACA was assayed immediately after final compounding, after a first incubation for nine days at 5° C., and after a second incubation for 21 days at either 22° C. or 5° C. The results are presented in Table 5.

TABLE 5

ACA of TNBP/cholate treated IGIV samples

Sample Point	ACA (CH$_{50}$/mL)
Intermediate Samples	
Initial sterile bulk	>100
Incubated 9 d. @ 5° C.	>100
Final Incubation	
21 d. @ 22° C.	49
21 d. @ 5° C.	71

In the initial sterile bulk sample, which was treated with TNBP/cholate at pH 7.0, the level of ACA was again greater than 100 units for the initial time point, confirming the observations noted in Table 3. Upon incubation at 5° C. for nine days, the ACA remained greater than 100 units. The final incubation step at either 5° C. or 22° C. shows that the reduction in ACA is dependent on temperature, with faster reduction in ACA observed at higher temperatures.

Effect of pH During Solvent/detergent Treatment on ACA

ACA levels were evaluated after incubation with TNBP/cholate at pH 5.8 because better viricidal activity was observed at pH values less than 6.0. Generally, the non-incubated sterile bulk samples of material incubated at pH 5.8 had lower ACA levels than the pH 7.0 samples, but the trend of lowering ACA upon incubation was repeated in the pH 5.8 samples. In fact, the ACA levels continue to decrease beyond the 21 day incubation in samples that initially had elevated ACA levels after incubation with TNBP/cholate at pH 5.8 (Table 6). As was previously noted for the samples incubated at pH 7.0, the lowering of ACA was not due to decreasing levels of aggregated IgG molecules because the material treated at pH 5.8 was essentially monomeric IgG prior to 22° C. incubation (HPLC analysis, sample A4, Table 8).

TABLE 6

Sample A4 - ACA upon extended incubation

Incubation at 22° C. (days)	CH$_{50}$/mL
0	122
10	73
19	55
25	56
28	45
30	40
34	39
41	33

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TABLE 6-continued

Sample A4 - ACA upon extended incubation

Incubation at 22° C. (days)	CH$_{50}$/mL
48	30
55	29

Similar results were achieved with samples formulated to 10% IGIV, 0.2 M glycine in the sterile bulk stage. Upon incubation at low ionic strength at pH 4.25 for 10 and 21 days, the levels of ACA were seen to decline in both 5% IGIV samples and 10% IGIV samples. (Table 7) The decrease in ACA can thus be observed over a range of ISG concentrations and over a range of pH values for the solvent/detergent treatment. (Tables 3, 5, 7) HPLC analysis (Table 8) of the sterile bulk samples presented in Table 7 confirmed that the elevated ACA levels were not due to aggregation of ISG molecules.

TABLE 7

ACA of samples treated with TNBP/cholate at pH 5.8

Sample	Sterile bulk (day zero) (CH$_{50}$/mL)	10 days incubation at 20–27° C. (CH$_{50}$/mL)	21 days incubation at 20–27° C. (CH$_{50}$/mL)
A1 (5% IGIV)	43	ND	10
A2 (5% IGIV)	31	14	15
A3 (5% IGIV)	44	15	12
A4 (5% IGIV)	122	73	55
B1 (10% IGIV)	>100	48	46
B2 (10% IGIV)	49	36	30
B3 (10% IGIV)	53	ND	37

Taken together, the above results suggest that ISG products which have been subjected to a solvent/detergent viral inactivation process resulting in an undesirable ACA increase can be made suitable for IV administration by incorporating an additional incubation step under the conditions described here to reduce the ACA to an acceptable level.

TABLE 8

HPLC Analysis of sterile bulk samples treated with TNBP/cholate at pH 5.8

Sample	Aggregate (%)	Dimer (%)	Monomer (%)	Fragment (%)
A2	0.140	0.00	99.86	0.00
A3	0.146	0.00	99.85	0.00
A4	0.124	0.00	99.88	0.00

CONCLUSION

The ACA increase resulting from the solvent/detergent treatment of the IGIV (antibody) solution appears to be an unavoidable secondary effect of TNBP/detergent treatment to inactivate viruses in the solution. I have discovered that by incubating the solution of IGIV at low pH (4.25) and low ionic strength (0.001M) for a relatively long period of time (at least about 10 days), the ACA gradually decreases over the period of incubation.

The prior art discloses a method of producing IGIV (the Tenold '608 patent) using low pH and low ionic strength.

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The Tenold '608 method omits the viral inactivation step, and thus avoids the problem of increased ACA, but the possibility of viral activity remains. Unlike Tenold, incubation is an essential aspect of the present invention for reducing the ACA.

The Neurath et al. '573 patent teaches the solvent/ detergent viral inactivation step. However, Neurath '573 does not mention controlling the pH and also does not mention any consequences of the process relating to ACA. Elevated ACA levels were detected at the sterile bulk stage of TNBP/cholate treated IGIV preparations. However, ACA levels decreased upon incubation for at least about 10 days at pH 4.25, low ionic strength, and not less than about 20° C. (See Tables 5–7) The prior art describes several approaches to lowering ACA levels of purified IgG preparations, including removal of IgG aggregates. (11) IgG aggregates have been shown to activate the complement system in vivo. (1) In the present invention, however, lowering of IgG ACA was not due to decreasing levels of IgG aggregates because these TNBP/cholate treated IGIV preparations contained low levels of aggregated IgG (as measured by HPLC, Tables 4, 8) prior to incubation under such conditions.

It would be desirable to produce substantially virus-free IGIV, but following the prior art results in a product with an unacceptable level of ACA. Note that Tenold '608 states that the product is substantially free of ACA, but use of the SD process in conjunction with Tenold '608 does result in high levels of ACA: experimental results reported here show that treating ISG solutions with the SD process and then formulation according to the Tenold '608 patent leads to a product with high ACA. (See Tables 1, 3, 5–7) The surprising finding reported here is that a follow-up (terminal) incubation step lowers the ACA of the solvent/detergent treated solution. The typical average observed ACA levels of 5% IGIV solutions treated according to the SD process and with or without the follow-up incubation are compared in the Figure. The present invention thus includes a previously unobserved method of reducing the ACA by incubating under controlled conditions of pH, temperature, and ionic strength for a period of time, thus allowing the product to be administered by intravenous injection.

Mitra '714 does not suggest the use of a S/D process but, instead, reports that a relatively brief incubation of an ISG product under similar conditions results in a substantially virus free preparation. (10) However, employing incubation under such conditions to provide a lowering of anticomplement activity is a novel application of these incubation conditions which were previously employed in the IGIV process for inactivation of enveloped viruses.

The newly developed IGIV process reported here, which includes an additional internationally accepted viral inactivation procedure (treatment with TNBP/cholate), generates IgG preparations which have low ACA levels and are suitable for IV administration. The major advantage is that an IGIV product with improved safety can be obtained by a two-step process that includes a TNBP/cholate treatment for viral inactivation and incubation under conditions that afford low ACA levels that are suitable for IV administration.

The above disclosure is intended to illustrate the invention, and it is thought variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention should be limited only by the claims below.

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What is claimed is:

1. A method of treating a solution of antibodies which may have virus activity, the method comprising

a) contacting the solution with a trialkylphosphate and a detergent under conditions sufficient to substantially reduce any virus activity and resulting in an increased level of anticomplement activity; and

b) then incubating the solution of step a) under conditions of controlled time, pH, temperature, and ionic strength, such that the increased anticomplement activity of the solution is reduced to an acceptable level suitable for intravenous administration.

2. The method of claim 1, wherein the anticomplement activity is reduced to less than about 60 CH_{50} units/mL.

3. The method of claim 1, wherein the solution comprises about 5% wt./wt. antibody and the anticomplement activity is less than about 45 CH_{50} units/mL.

4. The method of claim 3, wherein the solution comprises about 5% wt./wt. antibody and the anticomplement activity is less than about 30 CH_{50} units/mL.

5. The method of claim 1, wherein the solution comprises about 10% wt./wt. antibody and the anticomplement activity is less than about 60 CH_{50} units/mL.

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6. The method of claim 5, wherein the solution comprises about 10% wt./wt. antibody and the anticomplement activity is less than about 45 CH_{50} units/mL.

7. The method of claim 1, wherein the incubation is for at least about ten days.

8. The method of claim 1, wherein the pH is maintained within a range of about 3.5 to about 5.0.

9. The method of claim 1, wherein the temperature is maintained within a range of 2° C. to 50° C.

10. The method of claim 1, wherein the ionic strength is less than about 0.001 M.

11. The method of claim 1, wherein at least about 99% of the antibodies are monomeric.

12. The method of claim 1, comprising the further step of adjusting the tonicity of the solution to a physiologic value under such conditions that the ionic strength is not appreciably altered.

13. The method of claim 12, wherein the tonicity of the solution is adjusted by adding a carbohydrate to the solution.

14. The method of claim 13, wherein the carbohydrate used is maltose.

15. The method of claim 12, wherein the tonicity of the solution is adjusted to a range of about 230 to about 490 mosmol/kg solvent.

16. The method of claim 15, wherein the tonicity of the solution is adjusted to a range of about 274 to about 309 mosmol/kg solvent.

17. The method of claim 12, wherein the tonicity of the solution is adjusted by adding an amino acid to the solution.

18. The method of claim 17, wherein the amino acid used is glycine.

19. The method of claim 1, wherein the trialkylphosphate is tri-n-butyl phosphate and the detergent is selected from polysorbate 80 and sodium cholate.

20. The method of claim 1, wherein the solution has a pH between about 3.5 and about 6.0 during step a).

21. An intravenously injectable immune serum globulin preparation produced by the method of claim 1 and substantially free of lipid enveloped viruses, wherein the preparation has an ionic strength less than about 0.001 M, a pH between about 3.5 and about 5.0, an antibody concentration of about 5% wt./wt., and a maltose concentration of about 10% wt./wt.

22. The preparation of claim 21, wherein the pH is about 4.25.

23. An intravenously injectable immune serum globulin preparation produced by the method of claim 1 and substantially free of lipid enveloped viruses, wherein the preparation has an ionic strength less than about 0.001, a pH between about 3.5 and about 5.0, an antibody concentration of about 10% wt./wt., and a glycine concentration of about 0.2 M.

24. The preparation of claim 23, wherein the pH is about 4.25.

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EXHIBIT 11

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EXHIBIT 12

Anticomplementary Activity and the Safety of Intravenous Immunoglobulin

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ABSTRACT

Quality assurance release levels for anti-complementary (AC) activity of a non-modified intravenous immunoglobulin (IGIV, pH 4.25) were reviewed over a period of one year and nine months in an attempt to correlate the incidence of complement-mediated-type adverse reactions with the AC level. Over 200 lots and ten possible complement-mediated side effects were evaluated. No correlation was found. A similar evaluation was made of a prospective study in which three lots of IGIV, pH 4.25 (one with a low, one with a mid-range, and one with a high AC activity) were used; again no correlation was found. Finally, a comparison was made between the AC activity assay used to release IGIV, pH 4.25 and an alternative AC activity assay designed for a highly modified IGIV preparation. It was demonstrated that the AC activity assay designed for the modified preparation and its assay limits were unsuitable in attempting to define AC activity of the nonmodified preparation.

INTRODUCTION

The ability to fractionate human blood plasma into its component parts[1-5] enabled Barandun and his colleagues[6] in 1962 to investigate patients' intolerance to intravenous administration of the human gamma globulin fraction. Using a standard gamma globulin preparation intended for intramuscular use, they showed that only 13% of subjects without antibody deficiency developed a reaction on intravenous administration, whereas 92% of patients with antibody deficiency developed a reaction. The investigators felt it was unlikely that the reactions seen were due to direct antigen-antibody reaction—the recipients' antibodies reacting with antigens present in the gamma globulin concentrate—since patients with immune deficiency are unable to form antibodies.

They suggested that the reactions could be due to antigens in the recipient organism reacting with antibodies of the administered gamma globulin preparation, or that certain gamma globulin prepara-

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tions or certain lots of such preparations might have an intrinsic ability to activate the complement system when given intravenously. The latter mechanism might be explained by aggregated gamma globulins which fix complement by a similar mechanism to antigen-antibody complexes. Measures that might be taken to treat the standard gamma globulin preparation and render it less likely to spontaneously fix complement in the absence of antigen were presented.

A major characteristic of current gamma globulin preparations that have been rendered safe for intravenous use is either minimal anticomplementary (AC) activity or the absence of AC activity.[7-9] It is axiomatic, therefore, that an appropriate test for AC activity is included in the quality assurance battery required for release of the different intravenous immunoglobulin preparations.

We reviewed the data of certain anticomplementary activity assays used to release an intravenous immunoglobulin rendered safe for intravenous infusion by stabilization at pH 4.25 (IGIV, pH 4.25).[10] The AC activity was correlated against complement-type mediated adverse reactions that were reported spontaneously or observed during clinical investigations. We also evaluated an alternative assay specifically developed to measure AC activity of nonmodified intravenous immunoglobulin (IGIV, pH 4.25) to determine whether this assay and its limits could be used to assess the safety of the preparation for intravenous administration.

PATIENTS AND METHODS

Each patient in this study received infusions of an intravenous immunoglobulin fractionated from human blood plasma by the Cohn-Oncley fractionation method and then stabilized in solution at a pH of 4.25.[10] The number of lots used during the period of spontaneous reporting exceeded 200 and were freely available on the market. Clinical product research (PR) lots were used for patients in the surveillance study.

Two groups of patients were examined for possible complement-mediated adverse reactions. All untoward effects of the first group (group 1) considered attributable to administration of IGIV, pH 4.25 were spontaneously reported to Cutter Biological between the period of March 1986 and December 1987.

The second group (group 2) was comprised of 30 patients at two centers participating in a surveillance study. All 30 patients had primary immune deficiency (14 patients with X-linked agammaglobulinemia, 14 patients with common variable agammaglobulinemia, and two patients with Wiskott-Aldrich syndrome). The patients were randomly allocated to receive monthly infusions (400 mg/kg) of IGIV, pH 4.25. Each patient received four infusions from each of three different lots. A change of lot was made after the first four months and again after the second four months, with the third lot being administered in the last four months. The three lots used were chosen deliberately to have a high AC activity (PR 3025), a mid-range AC activity (PR 3032), and a low AC activity (PR 3055), as determined by in vitro AC activity Method 1. The AC activity of all three lots was within the acceptable AC activity range for this assay. A total of 342 infusions were administered. The average infusion time was 1.68 ± 0.49 (SD) hours; the average dosage administered was 399 mg/kg and the average volume given was 401 ml.

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Assays for Anticomplementary Activity

Method 1

Compliance with the AC activity specification in accordance with the Cutter Biological AC test is required for all lots of IGIV, pH 4.25 released to the market, as well as those used in the definitive surveillance study. This assay consists of addition of one volume of two CH_{50} units of guinea pig complement to an equal volume of sequential dilutions of test material in an appropriately buffered solution. The appropriate dilutions of hemolysin and complement are determined by titration prior to running the assay. The diluted test samples are allowed to react with complement for two hours at 37 °C. After incubation, sensitized sheep red blood cells are added and percent inhibition of hemolysis determined. The AC values are based upon the amount of test material that produces 50% inhibition of hemolysis. Reference standards are run with each assay to verify the performance of the test. The release limit for the test is less than 25 units CH_{50}/ml of product.

Method 2

For comparative purposes, complement assays were also performed on ten of the marketed lots, using a complement assay originally designed to assess complement activity of an intravenous immunoglobulin preparation rendered safe for intravenous administration by pepsin digestion. This assay uses one volume of 100 CH_{50} of guinea pig complement added to one volume of test material. Working dilutions of hemolysin and complement to be used in the assay are determined previously by titration. Three

volumes of appropriate buffer are added to the two volumes and the material is allowed to react for one hour at 37 °C. A control is run in which the volume of test material is replaced with one volume of appropriate buffer. After incubation the amount of complement present in each solution (control and test) is determined by titration. The control sample should contain no less than 85 CH_{50} units. The value of complement present in the test sample (CH_{50}/ml) is subtracted from the control value, and no more than 20 CH_{50} units may be inactivated. In order to ensure a control value above 85 CH_{50}, the initial complement titration is performed on complement that has been heated. Therefore more than 100 CH_{50} units are actually present in all of the test and control samples. The release limit for this test is considered to be less than 20 CH_{50} units complement per ml of product.

Reactions

A reaction possibly mediated by spontaneous activation of the complement system was deemed to have occurred if any one or more of the following symptoms were present either during or up to four hours postinfusion: warmth, flushing, headache, fever, chills, anxiety, malaise, faintness, nausea, vomiting, muscle pains, abdominal cramps, chest pain, chest tightness, dyspnea, wheezing, tachycardia, or rash.

RESULTS

Reactions—Group 1

During the period under study, there were a total of ten possible complement-mediated adverse reactions (Table I).

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Table I. Spontaneously reported possible complement-mediated adverse reactions in group 1, March 1986 through December 1987.

Lot Number	AC Activity (CH_{50}/ml) Method 1	Nature of Symptoms Reported
40B06	13.1	Chest tightness, dizziness, difficulty in breathing, lethargy
40B08	12.8	Headache after infusion
40P03	10.6	Vomiting, shortness of breath, abdominal pain after infusion
40P09	13.5	Generalized urticaria
40R02	14.2	Fever, increased pulse, chills, headache, general aches
40R03A	13.5	Flushing and fever after infusion
40S09	8.8	Dyspnea, hypotension, back pain, chills after infusion
40S10	8.5	(patient received two lots)
Not known	Not available	Headache, fever, malaise ten hours after infusion
Not known	Not available	Wheezing, chest tightness, dyspnea during rapid infusion
Not known	Not available	Wheezing, chest tightness, dyspnea during rapid infusion

AC = anticomplementary.

No lot had more than one such reaction; however, lot numbers for three reactions were not recorded and therefore no data are available. The reactions did not seem to occur with greater frequency with lots having higher AC activity.

During the one-year, nine-month study period, over 200 lots of IGIV, pH 4.25 were released. The distribution of the AC activity in these lots is shown in the figure. AC values ranged from 5.6 to 17.2 units CH_{50}/ml. Most of the lots (80%) fell within the range 8.0 to 14.0 units CH_{50}/ml.

Reactions—Group 2

There were a total of 15 reactions, four of which occurred during infusion (Table II). Although one of the four

reactions was considered severe, it did not necessitate discontinuing the infusion. Seven of the reactions, including the one classified as severe, were reported in association with the lot with low AC activity; all three reactions considered to be definitely related to the study drug infusion also occurred with this lot. There were five reactions with the lot with mid-range AC activity, and three reactions with the lot with high AC activity.

Comparison of Two Assays of Anticomplementary Activity

Ten lots of IGIV, pH 4.25 released for general usage were selected at random and their AC levels were compared using the two assays. The results are shown in Table III. It may be seen that according to

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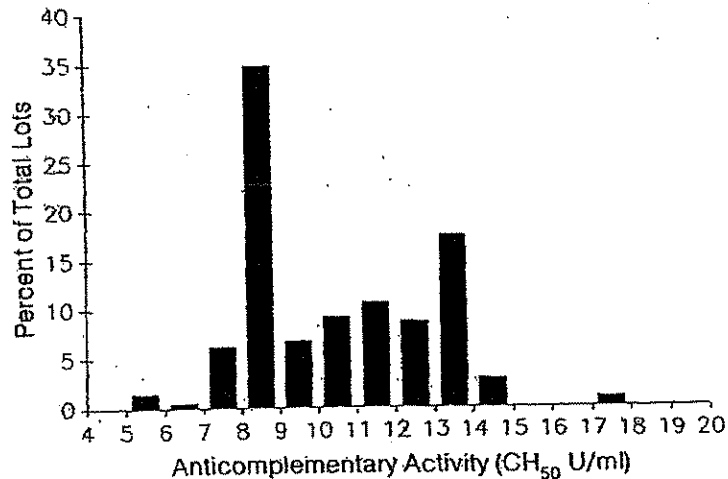† Reacti

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Figure. Anticomplementary activity of lots of IGIV, pH 4.25 between March 1986 and December 1987 (Method 1).

Table II. Possible complement-mediated reactions in group 2 during the surveillance study.

Lot Number	AC Activity (CH_{50}/ml) Method 1	Nature of Symptoms Reported
PR 3025	20.8	Rash after infusion
		Fever, lethargy after infusion
		Headache after infusion
PR 3032	15.6	Headache, seeing spots during infusion
		Abdominal cramps after infusion
		Headache after infusion
		Headache, abdominal cramps after infusion
		Headache after infusion
PR 3055	11.9	Chest tightness during infusion*
		Chest tightness, dyspnea during infusion* †
		Headache during infusion*
		Emesis after infusion
		Cough after infusion
		Emesis after infusion
		Emesis after infusion

AC = anticomplementary.
 * Only three of the reported reactions were considered definitely drug-related.
 † Reaction classified as severe.

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Table III. Comparison of anticomplementary (AC) activity in ten randomly selected lots of IGIV, pH 4.25 by two AC assay methods.*

Lot Number	AC Activity (CH_{50}/ml)	
	Method 1	Method 2
40B04	11.9	25.3
40B09	12.8	23.4
40B10A	11.9	24.7
40B11	13.5	28.6
40C04	17.2	28.5
40C05	14.3	25.5
40C07	10.4	21.6
40C08C	7.7	23.3
40C13A	8.6	26.0
40D02	8.2	25.1

* With Method 1, an acceptable AC level is considered to be below 25 units CH_{50}/ml, while with Method 2 it is considered to be below 20 units CH_{50}/ml. Pearson product-moment correlation = 0.56 ($P = 0.09$).

Method 1, all lots were considered to have acceptable AC activity, while with Method 2, all lots fell outside the limit for AC activity. None of these lots was associated with any spontaneously reported reactions even though one lot had the highest AC activity of all the lots released during the period under review.

The Pearson product-moment correlation between the results obtained with the two assays was found to be 0.56, which is not statistically significant ($P = 0.09$).

DISCUSSION

Spontaneously reported reactions are undoubtedly an underestimation, while the reverse is probably true for the definitive study in which every possible symptom ever associated with a complement-mediated reaction was taken to indicate such a reaction. Many of the reactions may have

been totally unrelated to the complement system, as is suggested by the absence of correlation between AC activity and the incidence of the reactions. In the definitive study, only one reaction was considered severe. There were no life-threatening reactions in either group of patients. There did not appear to be any correlation between the number and the severity of possible complement-mediated reactions with the level of AC activity in the lot used; in the definitive study there was almost a reverse correlation.

The data suggest therefore that the AC activity release limit and the assay used to measure the activity are reasonable and would not be associated with complement-mediated–type adverse reactions. The study showed that lots released in accordance with the specification limits defined by this assay did not show any significant incidence of complement-mediated adverse reactions.

The data also highlight the importance

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of using an AC assay that is specifically designed and validated for the sample (eg, intravenous immunoglobulin) that is to be assayed. The Cutter Biological assay of AC activity (Method 1) used to release lots of IGIV was designed for IGIV, pH 4.25. When the assay of AC activity designed for a pepsin-treated intravenous immunoglobulin (Method 2) was used, all IGIV, pH 4.25 lots tested in this manner had AC activities that were well above the release cutoff limit. Yet none of these lots failing the AC limit in accordance with this assay were associated with any adverse reactions. Finally, there was no correlation between the assay of AC activity by Method 1 and by Method 2 when both were used to test AC levels in IGIV, pH 4.25.

CONCLUSION

This study clearly demonstrates that the AC activity levels in IGIV, pH 4.25 are acceptable as defined by an assay validated to measure the levels in this preparation. This is also reflected in the safety of the preparation by the virtual absence of possible complement-mediated–type adverse reactions. The assay validated for a preparation finished by treatment with pepsin was unsuitable.

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ABSTRACT

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